Biodegradation of *p*-nitrophenol by microalgae

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Abstract

A study was made on the use of a mixed microalgal consortium to degrade *p*-nitrophenol. The consortium was obtained from a microbial community in a waste container fed with the remains and by-products of medium culture containing substituted aromatic pollutants (nitrophenols, chlorophenols, fluorobenzene). After selective enrichment with *p*-nitrophenol (*p*-NP), followed by an antibiotic treatment, an axenic microalgal consortium was recovered, which was able to degrade *p*-nitrophenol. At a concentration of 50 mg L^{-1}, total degradation occurred within 5 days. Two species, *Chlorella vulgaris* var. *vulgaris* f. *minuscula* and *Coenochloris pyrenoidosa*, were isolated from the microalgal consortium. The species were able to accomplish *p*-NP biodegradation when cultured separately, although *Coenochloris pyrenoidosa* was more efficient, achieving the same degradation rate as the original axenic microalgal consortium. When *Coenochloris pyrenoidosa* was associated with *Chlorella vulgaris* in a 3:1 ratio, complete removal of the nitro-aromatic compound occurred within three days. This is apparently the first report on the degradation of a nitro-aromatic compound by microalgae.

Abbreviations: *Chl. vulgaris* – *Chlorella vulgaris*, *C. pyrenoidosa* – *Coenochloris pyrenoidosa*, *p*-NP – *p*-nitrophenol

Introduction

Biodegradation of aromatic compounds has been studied with pure and mixed bacterial cultures (Hardman 1991; Alexander 1994). Bacterial degradation of nitrophenols has been widely studied and isolates included in the genera *Arthrobacter*, *Pseudomonas* and *Moraxella* have been found (Spain 1995). Although fewer studies have been carried out with microalgae, it has been reported that some eukaryotic algae and cyanobacteria are capable of biodegrading aromatic pollutants commonly found in wastewaters (Cerniglia et al. 1980; Craigier et al. 1965; Semple and Cain 1995; Semple et al. 1999). *Chlorella* sp. was found not only to decolourise certain azo-dyes, but to use them as carbon and nitrogen sources (Jingi and Houtian 1992). Semple and Cain (1996) reported degradation of phenol by *Ochromonas danica*. *Scenedesmus obliquus* was able to utilise naphthalenesulphonic acids as a source of sulfur (Luther 1990). Other authors have reported that three species of *Chlorella* were found to degrade pentachlorophenol in a light-dark photo-regime (Tikoo et al. 1997). However, the metabolic degradation pathways occurring in these organisms have not been widely addressed (Lovell et al. 2002). The combined action of different species of microalgae, or microalgae and other microorganisms, might provide a useful process for the elimination of these undesired compounds from the environment.

The aim of the study reported here was to obain and isolate a microalgal consortium effective in degrading *p*-nitrophenol and then to evaluate growth and degradation of the *p*-nitrophenol by the consortium and the individual species cells.

Materials and methods

Isolation of the degrading microalgal consortium

A mixed microbial community was recovered from a waste container, which was used, for several months, to keep liquid medium leftovers from laboratory experiments carried out with aromatic pollutants, mainly nitrophenols, chlorophenols and fluorobenzene. A 50-mL sample from the waste container was inoculated into 250 mL-flasks containing 50–100 mL of a sterile minimal salts medium (Caldeira et al. 1999). *p*-Nitrophenol (*p*-NP) was supplied to the liquid culture at a concentration of 10 mg L^{-1}. Cultures were incubated in a controlled growth chamber at 25 °C on a rotary shaker (Agitorb 300 EB, OMRON H7 ER) (100 ± 2 rpm) and 52.5 µmol of photons $s^{-1}m^{-2}$ (DataLogger LI-1000, LI-COR) photossinthetically active radiation (PAR) were provided by cool white fluorescent lamps set on 24 h-light photoregime. 50% of the culture was transferred to fresh medium at 15-day intervals, for a period of 3 months.

Axenic microalgae cultures

In order to obtain axenic microalgae cultures, to 100 mL culture, 1 mL filter sterilised antibiotic mixture (0.6 g of penicillin G and 1.0 g streptomycin sulphate in 200 mL distilled water) was supplied. The algal suspension was kept in this solution for 48 h, after which it was centrifuged, and the pellet was washed twice with sterile minimal salts medium. The pellet was further ressuspended in minimal salts medium containing yeast extract. Growth was promoted for 2 to 3 weeks at 25 °C, under a 24 h light regime with 52.5 µmol photon s^{-1} m^{-2} PAR. Samples of the microalgae cultures were spread onto plates of nutrient agar and onto minimal salts agar containing 10 mg L^{-1} *p*-NP, which were incubated at 25 °C, to verify the axenicity of the culture. Additionally, light microscopic analysis of culture samples was systematically carried out to ensure that bacteria were not present. Subsequently, growth of the axenic microalgal culture was carried out with 24 h-light incubation at 25 °C for 3 weeks. All the experiments were carried out using axenic cultures.

Isolation and purification of the microalgal consortium constituting species

Species present on the microalgal consortium were recovered in minimal salts medium agar plates supplied with *p*-NP. Biodegradation of *p*-NP was detected through the disappearance of the yellow colour, characteristic of this compound. Three morphologically distinct algae colonies were found. Each different colony was analysed for purity by spreading onto nutrient agar plates. The unialgal cultures obtained were inoculated into liquid minimal salts medium supplied with *p*-NP at 10 mg L^{-1} and incubated as described previously.

Biodegradation studies using the isolated microalgae species

Biodegradation of *p*-NP by the axenic microalgal consortium and by the unialgal species (*C. pyrenoidosa* and *Chl. vulgaris*) isolated from it was evaluated. A 1:1 mixture of the two species (same cell density of each unialgal culture); and a 2:1 and 3:1 mixture of *C. pyrenoidosa* and *Chl. vulgaris*, respectively, was used.

Culture conditions for degradation studies

Inocula from the above axenic unialgal cultures were used at a final cell density of approximately 1.8–2.0 × 10^6 cells mL^{-1}. *p*-NP was added to the minimal salts medium at 30 and 50 mg L^{-1}. The cultures were incubated at 25 °C on a rotary shaker with a 24 h light photoregime for up to 15 days. Inoculated medium free of *p*-NP and uninoculated medium with the nitro-aromatic compound served as controls. Each growth experiment was performed in triplicate. The same procedure was carried out for the biodegradation studies using unialgal species and the combined species.

Growth measurements

Cell density (number of cell mL^{-1}) was measured by microscope counting each culture twice in a Neubauer haemocytometer, from which growth curves were established for individual cultures. Biomass was also estimated as dry weight at the beginning and at end of experiments.

Nitrophenol measurements

An absorption spectrum of *p*-NP between 280–700 nm was established using a spectrophotometer linked to the computer software Spectro UV-1601PC. The maximum absorvance peak was found at 450 nm. Culture samples were centrifuged and supernatant absorvance was read at 450 nm after pH adjustment, whenever necessary. *p*-nitrophenol concentration of the sample was determined against a standard curve of *p*-NP (0–100 mg L^{-1}).

Results

Degradation of p-*nitrophenol by the isolated axenic microalgal consortium*

The absence of bacterial growth on the plates after the antibiotic treatment, and light microscopy analysis of culture samples, indicated that the microalgae cultures were axenic. Different types of microalgal cells were observed under light microscopy. The microalgal consortium was able to remove 30 and 50 mg L^{-1} *p*-NP from the culture medium. In the presence of 30 mg L^{-1} *p*-NP, after 4 days of incubation, approximately 2% of the compound remained in culture in two of the triplicates, and 10% remained in the other one (Figure 1). When *p*-NP concentration was increased to 50 mg L^{-1}, the lag phase increased to such an extent that the exponential growth phase was not initiated within 6 days. Approximately 5% of the compound was found in the cultures after 4 days of incubation in two of the triplicates and after 6 days on the other (Figure 2).

Isolation of the microalgae species within the consortium

Plating of the microalgal consortium on minimal salts medium agar plates, supplied with *p*-NP, revealed the presence of at least three different microalgae species, designated as A, B and C. These were transferred to fresh agar plates. The most abundant species, designated as colonial type A, was smaller than the other two and presented a dark green colour. The other two, differing in the apparent green colour, were denominated colonial types B and C. In order to identify the isolated microalgae species, morphological studies were conducted at the Department of Botany of the University of Coimbra. The characteristics observed

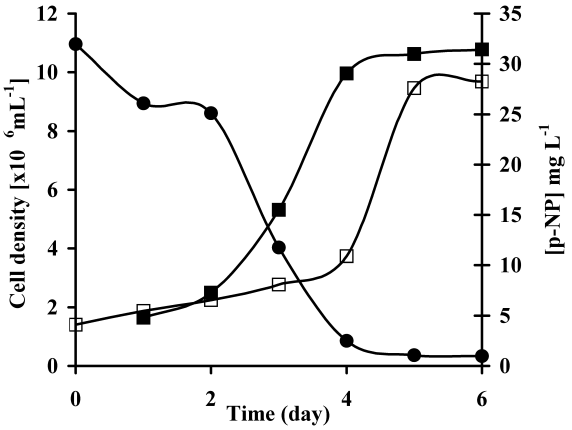

Figure 1. Growth of axenic microalgal consortium in 24 h light at 25 °C in an orbital incubator shaker at 100 rpm on 30 mg L^{-1} *p*-NP (□) and without *p*-NP (■), together with loss of *p*-NP (●). n = 3.

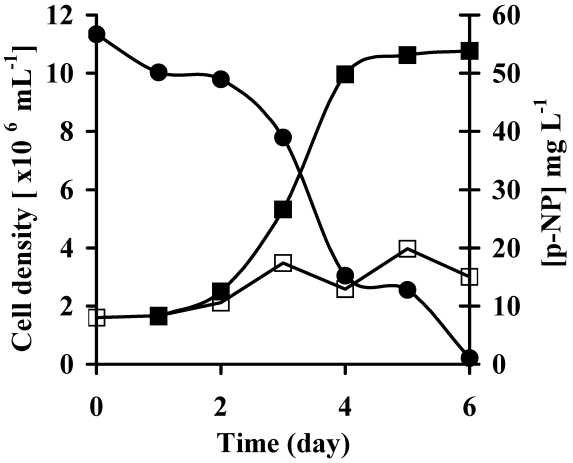

Figure 2. Growth of axenic microalgal consortium in 24 h light at 25 °C in an orbital incubator shaker at 100 rpm on 50 mg L^{-1} *p*-NP (□) and without *p*-NP (■), together with loss of *p*-NP (●). n = 3.

led to the following identification: colonial type A – *Chlorella vulgaris* Beijerink var. *vulgaris* f. *minuscula* Andreeva (*Chl. vulgaris*) and colonial types B and C – *Coenochloris pyrenoidosa* Korsikov (*C. pyrenoidosa*).

p-*Nitrophenol degradation by the isolated unialgal species*

Unialgal cultures named A, B and C were tested for the ability to degrade *p*-NP supplied at 50 mg L^{-1} (Figure 3). Cultures B and C, both identified as *C. pyrenoidosa*, were able to remove all the supplied *p*-NP from the culture in 5 days. The same pattern was observed with the microalgal consortium. How-

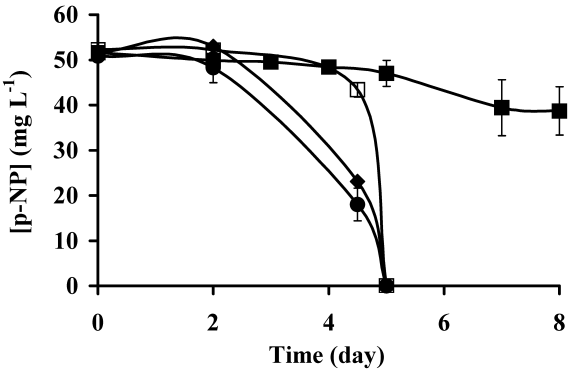

Figure 3. Degradation of 50 mg L^{-1} *p*-NP by the microalgal species, A (■), B (♦) and C (●), and by the axenic microalgal consortium (□). Mean ± S.D., n = 3.

ever, after 8 days of incubation of culture A, identified as *Chl. vulgaris*, about 77% of *p*-NP still remained in the culture medium (Figure 3). The pH affected greatly the degrading capacity of these microalgae species. In a 15-day experiment conducted on non-buffered media, where there was a pH increase from 7.0 up to 9.2, biodegradation of *p*-NP did not occur (data not shown).

p-*Nitrophenol degradation by different combinations of unialgal species*

The degradation of *p*-NP by cultures established with different proportions of each unialgal species was evaluated. The kinetics of degradation of *p*-NP seemed to be dependent of the initial proportion of each species (Figure 4). *Chl. vulgaris* took longer than *C. pyrenoidosa* to accomplish the removal of the nitro-aromatic compound. While *C. pyrenoidosa* unialgal cultures took 5 days to degrade *p*-NP at 50 mg L^{-1}, a predominant *C. pyrenoidosa* culture, with three times higher cell density than the other microalgae species, took only 3 days to degrade the same amount of *p*-NP. The cell mixture with two times higher cell density of *C. pyrenoidosa* was able to degrade 50 mg L^{-1} of *p*-NP within 4 days. The mixture that had similar cell density of each isolated microalgal species required 5 days to remove the same amount of *p*-NP. All the triplicates within each set of experiments presented the same degradation profile. A comparison between the degrading capacity of the original microalgal consortium, the independent unialgal species and the different mixtures of the isolated species is presented in Table 1.

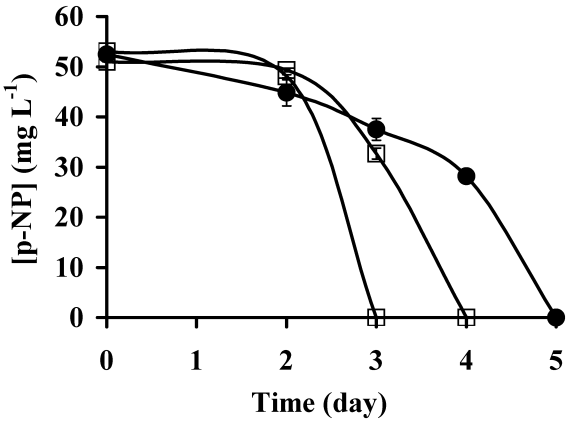

Figure 4. Degradation of 50 mg L^{-1} *p*-NP by different mixtures of two microalgal species: *Chl. vulgaris* and *C. pyrenoidosa* (1:1) (●), *Chl. vulgaris* and *C. pyrenoidosa* (1:2) (□), *Chl. vulgaris* and *C. pyrenoidosa* (1:3) (■). Mean ± S.D., n = 3.

Discussion

The axenic microalgal consortium recovered from the waste container was capable of degrading *p*-NP at 30 and 50 mg L^{-1}. Exponential growth of the microalgae cultures was always initiated after removal of *p*-NP from the medium, suggesting that only after the disappearance of the pollutant the microalgae find the appropriate conditions to grow. The duration of the lag phase was longer in the presence of higher initial *p*-NP concentrations. Different degradation rates were observed within the triplicates in the experiments carried out with the microalgal consortium, although all followed similar trends. This could be due to variations on the ratio of the species present within the consortium. At least two distinct microalgae species composed the initial microalgal consortium. Isolates recovered from that consortium, identified as *Chl. vulgaris* and *C. pyrenoidosa*, were capable of degrading *p*-NP, both individually and in different combinations. According to the species identification report, cultures B and C were the same species – *C. pyrenoidosa*, and therefore identical biodegradation profiles were expected, which was in fact confirmed by the experiments carried out with the unialgal cultures. *Chl. vulgaris* took longer than *C. pyrenoidosa* to accomplish the removal of the nitro-aromatic compound, which suggests that it has less relevance in the degradation process. Biodegradation experiments were also carried out with *Chl. vulgaris* and *C. pyrenoidosa* associated in different proportions. The removal of 50 mg L^{-1} *p*-NP from the culture medium was accomplished faster when higher amounts of *C.*

Table 1. *p*-NP removal capacity and lag phase duration of the initial microalgal consortium, the unialgal cultures and the different combinations of unialgal cultures (1:1, 2:1, and 3:1).

	$mg^{-1}\ L^{-1}\ d^{-1}$	duration (days)
Initial microalgae consortium	8.3	6
C. pyrenoidosa	10.0	4
Chl. vulgaris	2.0*	4
C. pyrenoidosa and *Chl. vulgaris* (1:1)	10.0	2
C. pyrenoidosa and *Chl. vulgaris* (2:1)	12.5	2
C. pyrenoidosa and *Chl. vulgaris* (3:1)	16.5	2

*based on 33% degradation which occurred within 8 days

pyrenoidosa was present in the association. As in the unialgal degradation studies, the *Chl. vulgaris* did not seem to account significantly for the disappearance of *p*-NP. In fact, the removal capacity of *p*-NP was always higher when *C. pyrenoidosa* predominated. Although *C. pyrenoidosa* on its own was able to achieve similar degradation rates as the original microalgal consortium, its degradation activity was higher when in the presence of *Chl. vulgaris*, suggesting that the consortium composition determines the rate of degradation of the organic compound. Mutualistic interactions between microbial strains that degrade xenobiotics may be involved in the degradation process, as it is reported for bacteria (Bull and Slater 1982).

Few biodegradation studies on phenolic compounds with microalgae have been described. The eukaryotic alga *Ochromonas danica* was reported by Semple and Cain (1996) to grow heterotrophically on phenol as the sole source of carbon up to concentrations of 4 mM, although the authors did not rule out the possibility of activity for internal bacteria in this organism, which is known to be capable of phagotrophy. Shashirekha et al. (1997) have shown that the marine cyanobacterium *Phormidium valderianum* BDU 30501 was able to tolerate and grow at a phenol concentration of 50 mg L^{-1} and remove 38 mg L^{-1} within a retention period of 7 days. We have found no studies of biodegradation of *p*-NP by microalgae species, but we have accomplished the removal of 50 mg L^{-1} within a minimal period of 3 days, when *C. pyrenoidosa* was associated with *Chl. vulgaris* in the proportion of 3:1. Both the axenic microalgal consortium and the unialgal species were able to remove *p*-NP from the medium, which extends the knowledge on the ability of such organisms to participate in the removal of such compounds from the environment.

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